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Magnetic properties of oxyhemoglobin

(hemoglobin/iron electronic state)

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ABSTRACT **When the magnetic susceptibility of frozen aqueous solutions of human oxyhemoglobin was measured in the range between 25 and 250 K, it showed a temperature-dependent behavior typical of a thermal equilibrium between a ground singlet state and an excited triplet state for two electrons per heme, the energy separation being $|2J| = 146$ cm^{-1}. By contrast, within the same temperature range, carboxyhemoglobin was found to be diamagnetic, as already reported.**

Since the first magnetic studies by Pauling and Coryell (1), the electronic state of iron and oxygen in oxyhemoglobin (HbO_2) has been the subject of continuous debate. Five different models for O_2 binding in HbO_2 have been proposed on the basis of various theoretical interpretations of the available experimental evidence (2–6).

The presence of unpaired electrons in the iron-oxygen bond has been inferred from x-ray fluorescence spectra (7), from spectroscopy data and analogy with cobalt complexes (8, 9), and from Mössbauer (10) and Raman (11) spectra.

Despite this, in the majority of papers in this field, it is assumed that HbO_2 and carboxyhemoglobin (HbCO) are diamagnetic, which has precluded definite assignment of the electronic state of the site and has strongly biased the discussion.

The present work is intended to remove this bias and to produce new experimental evidence about the magnetic state of HbO_2, which is at variance with the commonly accepted behavior as cited above. Our experimental method, based on our oscillating sample (12) superconducting magnetometer (13), differs from previous methods mainly in the fact that we scan a wide temperature range, between 25 and 250 K, rather than take measurements only at a single temperature. This procedure allows us to resolve small paramagnetic contributions on top of the dominating background of the frozen protein solution without resorting to less direct subtraction procedures.

MATERIALS AND METHODS

Native and stripped human HbO_2 was prepared according to the method of Benesch *et al.* (14). Sample concentrations were in the range of 10 mM in heme. Measurements always were made on fresh samples, by the following procedure. Within 24 hr after blood was drawn from one of us (F.M.), part of the sample at proper concentration and pH was saturated with air or pure O_2 and brought to liquid helium temperature in the susceptometer, and its temperature-dependent susceptibility was measured. The rest of the sample was stored at 4 °C in equilibrium with air. The next day it was equilibrated with pure CO and then inserted in the susceptometer for measurements. Optical spectra of the samples were recorded by a Beckman DK-2A spectrophotometer before and after the susceptibility measurement. The concentration of methemoglobin (MetHb) in the samples was estimated to be less than 3% in all cases. To avoid any possible interferences from MetHb, we added KCN to stripped HbO_2 to stabilize the MetHb in the low-spin form (15) at any temperature. Measurements were performed on several different preparations of Hb. The temperature-dependent susceptibility was measured at least twice on each sample.

RESULTS AND DISCUSSION

To emphasize the small differences between HbO_2 and HbCO samples, all the data were normalized to give the same extrapolation for infinite temperature, which was set as the value extracted from the data of Havemann (16) for HbCO. Typical results for stripped HbO_2 and HbCO equilibrated with air, O_2 at 1 atm, and CO at 1 atm, are presented in Fig. 1.

There is a striking difference in behavior. The stripped HbCO showed a weak Curie law paramagnetic contribution which is quantitatively accounted for by the presence of 0.85% MetHb in the low-spin state.

Native HbCO gave similar results, with an even smaller paramagnetic contribution. We conclude that both native and stripped HbCO are diamagnetic. We also conclude that (*i*) no sample density effects interfered with the volume susceptibility measurements within our resolution and (*ii*) MetHb, if any, must stay in the same low-spin state in the whole temperature range scanned and strictly obey a simple Curie law.

The temperature dependence of the magnetic susceptibility of the frozen solutions of stripped HbO_2 was strikingly different from that of HbCO.

To fit the data we needed a composite temperature-dependent behavior that allows for the presence of a Curie law paramagnetic component plus a paramagnetic contribution nonlinear in inverse temperature, representing a thermal equilibrium between a ground singlet state and an excited triplet state. The Curie law paramagnetic contribution is easily assigned to the paramagnetism of the oxygen in solution and to an amount of low-spin MetHb smaller than 3%. To account for the nonlinear paramagnetic component we have assumed that two unpaired electrons are present in the iron–oxygen bond and their spins pair off in a singlet state at low temperature (<40 K) and thermally populate a triplet state at higher temperature. To describe this behavior we have used the appropriate theoretical relationships for two spins with $S_1 = S_2 = \frac{1}{2}$ and singlet-triplet separation of -2J, as given in textbooks (17). The full curves in Fig. 1 represent a computer-performed least-square best fit to the data, with J and the Curie constant of the paramagnetic component left as free parameters. For the singlet-triplet half separation of one spin pair per heme we get $J = -72$ cm^{-1}.

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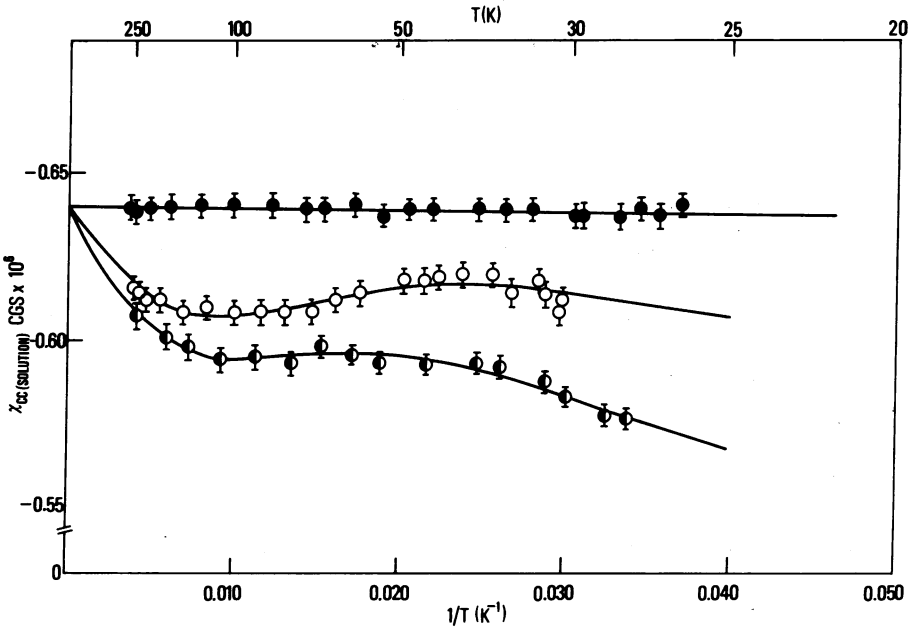

FIG. 1. Magnetic susceptibility of ligated stripped human hemoglobin plotted against reciprocal of inverse temperature, shown as mean. ○, HbO_2 equilibrated with air, pH 7.2; heme concentration 9.6 mM. ◐, $HbO_2 + CN^-$, pH 7.4; heme concentration, 11.1 mM; CN^-, 2 mM; equilibrated with 1 atm O_2. —, best-fit curve for a Curie law paramagnetic component plus the contribution from a coupled spin pair with $S_1 = S_2 = ½$, g = 2.00, TIP = 0, and $J = -72\ cm^{-1}$ for HbO_2 or $J = -73.5\ cm^{-1}$ for $HbO_2 + CN^-$. ● HbCO equilibrated with 1 atm CO, pH 7.2; heme concentration, 10.6 mM.

When we attempted a similar fit for the HbCO data, we only found a lower limit in the form $|J_{CO}| \geq 400\ cm^{-1}$.

Data on native HbO_2 and on stripped HbO_2 containing CN^- gave similar results, with $J = -72.5\ cm^{-1}$ and $J = -73.5\ cm^{-1}$, respectively. The differences in J among the three samples are not significant in the resolution of our fitting procedure. The paramagnetic component was larger in the sample containing CN^- because the sample had been equilibrated with pure O_2 rather than with air. When this is accounted for, the residual MetHb concentration is similar for both oxygenated samples, about 3%.

The measurements on stripped HbO_2 with added CN^- were performed because CN^- is known to bind strongly to MetHb, stabilizing it in a low-spin state within the whole temperature range explored (15). Thus, no composite temperature-dependent behavior can be expected from any MetHb present in the

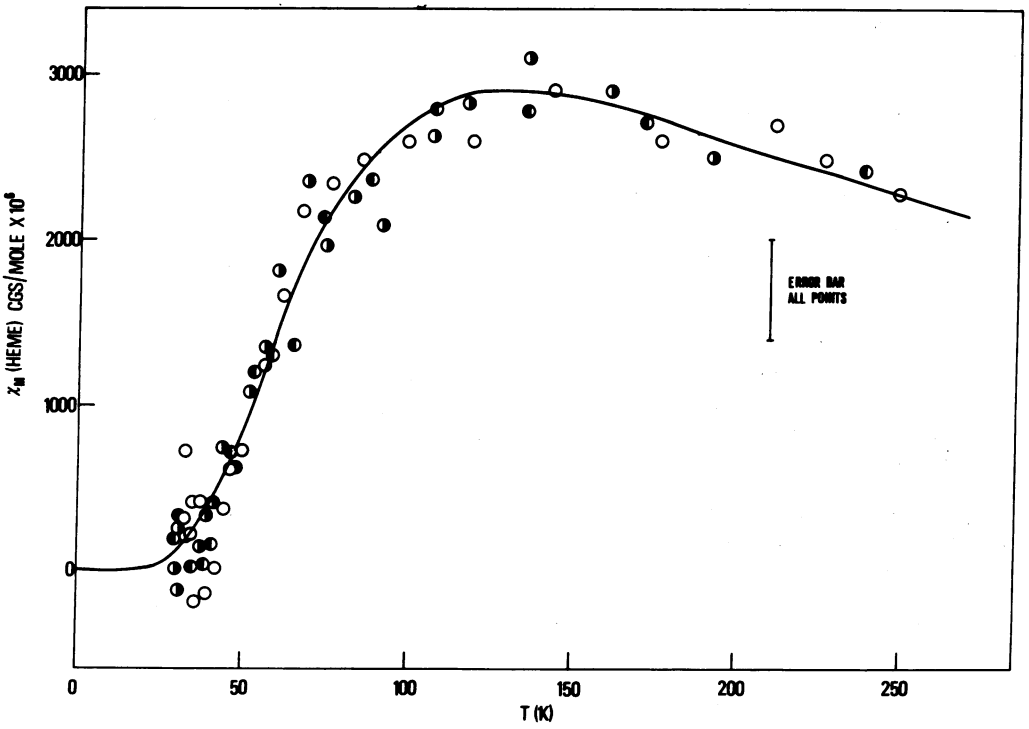

FIG. 2. Deviation from Curie law and diamagnetic background for the oxygenated samples, expressed as the dependence of the suceptibility per heme as extracted from the display of data of Fig. 1, and for native HbO_2. ○, stripped HbO_2. (◐), stripped $HbO_2 + CN^-$. ◑, native HbO_2; 11.2 mM in heme, pH 7.2, equilibrated with 1 atm O_2. —, theoretical curve for $S_1 = S_2 = ½$, g = 2.00, TIP = 0, and $J = -73\ cm^{-1}$.

sample. The agreement between the data for the CN^- sample and the other data is taken as a crucial test that the temperature-dependent behavior observed in HbO_2 solutions is indeed due to the iron–oxygen complex. We can also rule out a temperature-dependent dissociation of diamagnetic HbO_2 to deoxyHb plus O_2. Indeed, we obtained the same results for the nonlinear component, while the oxygen affinity increased by at least a factor of 5 on going from native to stripped Hb samples and changing the oxygen concentration by a factor of 5 (equilibrating with air or with pure O_2) (see also Fig. 2). Moreover, no sign of dissociation could be detected in the optical spectra.

To emphasize the agreement between the three oxygenated samples and the satisfactory fit with the theoretical curves, we show in Fig. 2 the experimental data for all three kinds of samples expressed as molar susceptibility per heme, after subtraction of the diamagnetism of the solution and of the Curie law paramagnetic component, as given by the computer fitting procedure. It is apparent that the characteristic nonlinear behavior of the atomic susceptibility is due to a triplet state that becomes thermally populated from a singlet state. The full curve represents the corresponding theoretical curve with $J = -73\ cm^{-1}$, which fits well (within experimental error) all the data from different samples.

From our experimental evidence we conclude that, at variance with what is widely accepted, HbO_2 is not diamagnetic above about 50 K and that the iron–oxygen complex contains a coupled $S = \frac{1}{2}$ spin pair.

In regard to the electronic state of the iron–oxygen bond, as suggested by the present data, we notice that an $S = \frac{1}{2}$ pair in the bond would be consistent with the assignment by Barlow *et al.* (18) of a bond order of 1.5 for the O–O bond in HbO_2. It is also in accord with the enhanced O_2 uptake by model iron porphyrins in polar aprotic solvents (19, 20). If we assign one electron to the O–O bond and the other to the Fe–O bond, our data require an antiferromagnetic coupling between their spins. This is consistent with the Weiss model (4), which gives $Fe^{3+}O_2^-$. Whatever the localization of these two electrons, the iron would still be low-spin, with at most one unpaired electron. This would still agree with the view of the iron being in the low-spin state when ligated (21).

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